IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Reissue Application of :

Tohru UEDA et al. : ATTN: APPLICATION BRANCH

U.S.Patent No. 5,026,835 :

Issued June 25, 1991 :

For : PYRIMIDINE 2'-METHYLIDENE NUCLEOSIDE COMPOUNDS

DECLARATION UNDER 37 CFR 1.132

Honorable Commissioner of
Patents and Trademarks,
Washington, D.C. 20231

Sir:

I, Akihiro Fujii declare :

That I am a citizen of Japan, whose full post office address is Yoshitomi Pharmaceutical Industries, Ltd. c/o Tokyo Research Laboratories, 7-25, Koyata 3-chome, Iruma-shi, Saitama 358, Japan.

That my education and employment history is as follows:

I was graduated from Tokyo University, Faculty of Science in March, 1983. I was employed by Yoshitomi Pharmaceutical Industries, Ltd. in April, 1983. I have been engaged in the research and development of antitumor agents at the Tokyo Research Laboratories of said company.

That I am a co-inventor in this application;

That I am a member of the Japanese Cancer Association, and papers in the name of my colleagues and myself are;

· Takenuki, K., Matsuda, A., Ueda, T., Sasaki, T., Fujii, A.,

ATTACHMENT G

and Yamagami, K., Design, synthesis, and antineoplastic activity of 2'-deoxy-2'-methylidenecytidine, J. Med. Chem., 1988, 31, 1063-1064.

- Yamagami, K., Fujii, A., Arita, M., Okumoto, T., Sakata, S., Matsuda, A., Ueda, T., and Sasaki, T., Antitumor activity of 2'-deoxy-2'-methylidenecytidine, a new 2'-deoxycytidine derivative, Cancer Res., 1991, 51, 2319-2323.

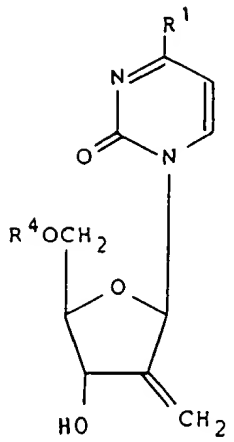
- Miyashita, T., Ashida, N., Kondoh, K., Sakata, S., Machida, H., Fujii, A., Ueda, T., and Matsuda, A., Synthesis and antitumor activity of acyl and benzyl types of prodrugs of 2'-deoxy-2'-methylidenecytidine, Nucleosides and Nucleotides, 1992, 11, 495-513.

That the following comparative experiment was conducted under my supervision wherein it has been demonstrated that the compound of the present invention has superior antitumor activity as compared with the compound (2'-deoxy-2'-methylidenecytidine=DMDC) of JP-A-63 258 818;

That the following demonstrates the above facts;

Comparative Experiment

Table



Compound	Structure R^1	Structure R^4	Antitumor activity Suppression of tumor growth (in vivo) (relative potency to DMDC)*
DMDC	$-NH_2$	-H	1.00
Y-1	$-NH_2$	$-COC_9H_{19}$	4.30
Y-2	$-NH_2$	$-COC_{11}H_{23}$	4.20
Y-3	$-NH_2$	$-COC_{13}H_{27}$	4.43
Y-4	$-NH_2$	$-COC_{15}H_{31}$	3.57
Y-5	$-NH_2$	$-COC_{17}H_{35}$	3.43
Y-6	$-NHCOC_9H_{19}$	-H	3.30
Y-7	$-NHCOC_{11}H_{23}$	-H	3.57
Y-8	$-NHCOC_{13}H_{27}$	-H	3.03
Y-9	$-NHCOC_{15}H_{31}$	-H	4.67
Y-10	$-NHCOC_{16}H_{33}$	-H	4.30
Y-11	$-NHCOC_{17}H_{35}$	-H	3.97

*: Each compound (dose converted to DMDC) was orally administered to a nude mouse implanted with human LX-1 lung cancer for 5 consecutive days, and suppressive effect on tumor growth was examined by measuring the tumor volume on Day 7. The value was expressed as relative potency to DMDC.

<Preparation Method>

Compounds Y-1, Y-5, Y-6, and Y-11 correspond to the compounds prepared in Examples 4, 3, 2 and 1, respectively. Compounds Y-3 and Y-4 are disclosed in page 15, lines 5 and 6 of the Specification. The preparation method of other compounds (Y-2, Y-7～10) are as follows.

(1) 2'-deoxy-2'-methylidene-5'-O-lauroylcytidine (Y-2)

2'-Deoxy-2'-methylidenecytidine · HCl, 1.5 g, was dissolved in 17 ml of dimethylformamide, and to the solution was added dropwise 1.97 g of lauroyl chloride under ice-cooling, followed by stirring for 27 hours. After the reaction, 20 ml of methanol was added to the reaction mixture, and further, 7 ml of a saturated aqueous solution of sodium bicarbonate was added thereto. After the solvent was distilled off, the resulting oily substance was treated with silica gel chromatography (elution solvent-methanol:chloroform=1:20), and the solvent was distilled off to give 860 mg (38 %) of the objective compound as colorless amorphous solid.

NMR (DMSO-d_6) δ ppm :

0.86 (3H, t, J=5.0Hz, CH_3)

1.03-1.76 (18H, m, $(CH_2)_9$)

2.32 (2H, t, J=6.8Hz, $-COCH_2-$)

3.53-3.92 (1H, m, H-4')

4.06-4.63 (3H, m, H-3' and H-5')

5.18 (1H, m, H-2"a)

5.36 (1H, m, H-2"b)

5.72 (1H, d, J=7.5Hz, H-5)

5.78 (1H, d, J=6.3Hz, 3'-OH)

6.54 (1H, s, H-1')

7.24 (2H, br.s, NH_2)

7.39 (1H, d, J=7.5Hz, H-6)

Optical density (α_D):

-20.4 ° (c=0.1, in $CHCl_3$)

Elemental analysis :

(as $C_{22}H_{35}N_3O_5 \cdot 1/2\ H_2O$)

Calculated (%) C : 61.37, H : 8.43, N : 9.76

Found (%) C : 61.49, H : 8.39, N : 9.83

MS (70eV) m/z:421 (M+)

IR (KBr): 3348, 3205, 1736, 1651 cm^{-1}

(2) 2'-deoxy-2'-methylidene-N^4-lauroylcytidine (Y-7)

2'-Deoxy-2'-methylidenecytidine, 1.4 g (5.1 mmol), was dissolved in 30 ml of pyridine, and to the solution was added dropwise 1.4 g of trimethylsilyl chloride under ice-cooling. After allowing the mixture to become room temperature, the mixture was stirred for 15 minutes, and then 1.2 g (5.5 mmol, 1.1 eq.) of lauroyl chloride was added dropwise to the mixture under ice-cooling, followed by stirring at room temperature for 2 hours. After the reaction, a lump of ice was added to the reaction solution, and the mixture was stirred for 5 minutes. Then, 2 ml of concentrated ammonia water was added to the solution, followed by stirring for 15 minutes. The reaction

solution was diluted with chloroform, partitioned, and washed with saturated brine, and the organic layer was dried and concentrated under reduced pressure. The residue was treated with silica gel chromatography to give 1.1 g (2.6 mmol) of the foamy objective compound.

NMR (DMSO-d_6) δ ppm :

8.13 (1H, d, J=7.6Hz, 6-H)

7.21 (1H, d, J=7.6Hz, 5-H)

6.56 (1H, s, 1'-H)

5.65 (1H, d, J=4.6Hz, 3'-OH)

5.34 (2H, br.s, 2'-methylene)

4.99 (1H, br.t, 5'-OH)

4.53 (1H, m, 3'-H)

3.50-3.91 (3H, m, 4',5'-H)

2.40 (2H, br.t, CO-$\underline{CH_2}$)

1.05-1.81 (18H, m, $(\underline{CH_2})_9$)

0.86 (3H, br.t, CH_3)

Optical density :

-1.3 (c=0.5, in methanol)

Elemental analysis :

(as $C_{22}H_{35}N_3O_5$)

Calculated (%) C : 62.69, H : 8.37, N : 9.97

Found (%) C : 62.58, H : 8.46, N : 9.54

UV λ max (in methanol)

248.5, 301.2 (nm)

EI-MS (m/z) 421 (M+), 403 (M^+-H_2O), etc.

(3) 2'-deoxy-2'-methylidene-N^4-myristoylcytidine (Y-8)

The title compound was obtained in the same manner as described in (2) except the use of myristoyl chloride instead of lauroyl chloride.

NMR (DMSO-d_6) δ ppm :

0.86 (3H, t, J=5.0Hz, CH_3)

1.00-1.37 (22H, m, $(CH_2)_{11}$)

2.48 (2H, t, J=5.0Hz, $-COCH_2-$)

3.58-3.82 (3H, m, H-4' and H-5')

4.40-4.70 (1H, m, H-3')

4.99 (1H, t, J=5.0Hz, 5'-OH)

5.35 (2H, m, H-2")

5.65 (1H, d, J=6.3Hz, 3'-OH)

5.56 (1H, s, H-1')

7.21 (1H, d, J=7.5Hz, H-5)

8.10 (1H, d, J=7.5Hz, H-6)

Optical density (α_D):

-5.5° (c=0.1, in methanol)

Elemental analysis :

(as $C_{24}H_{39}N_3O_5 \cdot 1H_2O$)

Calculated (%) C : 61.65, H : 8.84, N : 8.99

Found (%) C : 61.98, H : 8.30, N : 9.01

UV λ max (in methanol):

300.4, 249.8 (nm)

MS (70eV) m/z:449 (M+)

IR (KBr): 3327, 1703, 1649 cm^{-1}

Melting point : 123 ℃

(4) 2'-deoxy-2'-methylidene-N^4-palmitoylcytidine (Y-9)

The title compound was obtained in the same manner as described in (2) except the use of palmitoyl chloride instead of lauroyl chloride.

NMR (DMSO-d_6) δ ppm :

0.86 (3H, t, J=5.0Hz, CH_3)

1.05-1.70 (26H, m, $(CH_2)_{13}$)

2.40 (2H, t, J=7.5Hz, $-COCH_2-$)

3.55-3.85 (3H, m, H-4' and H-5')

4.43-4.67 (1H, m, H-3')

4.90-5.18 (1H, m, 5'-OH)

5.36 (2H, br.s, H-2")

5.55-5.85 (1H, m, 3'-OH)

6.59 (1H, s, H-1')

7.23 (1H, d, J=7.5Hz, H-5)

8.13 (1H, d, J=7.5Hz, H-6)

Optical density (α_D):

-3° (c=0.1, in methanol)

Elemental analysis :

(as $C_{26}H_{43}N_3O_5$)

Calculated (%) C : 65.38, H : 9.07, N : 8.80

Found (%) C : 64.78, H : 9.10, N : 8.41

UV λ max (in methanol):

299.8, 249.0 (nm)

MS (70eV) m/z: 477 (M+)

IR (KBr): 3320, 1702, 1650 cm^{-1}

Melting point : 120-121 ℃

(5) 2'-deoxy-2'-methylidene-N^4-heptadecanoylcytidine (Y-10)

The title compound was obtained in the same manner as described in (2) except the use of heptadecanoyl chloride instead of lauroyl chloride.

NMR (DMOS-d_6) δ ppm :

0.86 (3H, t, J=5.0Hz, CH_3)

1.09-1.81 (28H, m, $(CH_2)_{14}$)

2.39 (2H, t, J=7.0Hz, $-COCH_2-$)

3.53-3.90 (3H, m, H-4' and H-5')

4.53 (1H, m, H-3')

4.99 (1H, br.t, 5'-OH)

5.34 (2H, m, H-2")

5.65 (1H, d, J=6.3Hz, 3'-OH)

6.56 (1H, s, H-1')

7.21 (1H, d, J=7.5Hz, H-5)

8.13 (1H, d, J=7.5Hz, H-6)

Optical density (α_D):

-19 ° (c=0.1, in chloroform)

Elemental analysis :

(as $C_{27}H_{45}N_3O_5 \cdot 1/2\ H_2O$)

Calculated (%) C : 64.77, H : 9.26, N : 8.39

Found (%) C : 64.77, H : 9.00, N : 8.37

UV λ max (in methanol):

300.4, 248.6 (nm)

MS (70eV) m/z: 491 (M+)

IR (KBr): 3339, 1703, 1651 cm^{-1}

Melting point : 122-124 ℃

<Evaluation of Antitumor Activity>

Fragments (2×2×2 mm) of LX-1 (human lung cancer tumor) were implanted s.c. into the back of BALB/C-nu/nu mice. When tumor volume reached 100-500 mm^3, the mice were randomly allocated to several experimental groups consisting of 4-5 animals each, and compounds (drugs) were administered p.o. once a day for 5 days (Days 0-4). Tumor diameters were measured with calipers, and the tumor volume (V) was calculated as

$$V = \frac{L \times W^2}{2}$$

where L and W are the long and short diameters (mm) of the tumor mass.

Each tumor volume was calculated and expressed as relative tumor volume

$$V_n / V_0$$

where V_n is the tumor volume on Day n and V_0 is the initial tumor volume at the time when the treatment was started (Day 0). The mean value of V_n / V_0 (RV) was calculated for each group and drug efficacy was expressed as the percentage of the RV of

the control group :

$$T/C\ (\%) = \frac{\text{RV of the test group}}{\text{RV of the control group}} \times 100$$

In a similar manner, the efficacy of the compounds was evaluated by relative potency to DMDC the efficacy of which was taken as 1.00.

As shown in the above Table, the antitumor activity of the compound of the present invention is superior to that of DMDC as disclosed in JP-A-63 258 818. The results obtained show the surprising effect of the present invention.

For the experiment, Dr. Shinji Sakata of Yamasa Corporation provided samples of DMDC, Y-1, Y-5, Y-6, and Y-11, and Dr. Masafumi Arita of Yoshitomi Pharmaceutical Industries, Ltd. provided the rest of the samples.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed at Saitama, Japan on this 23rd day of July , 1993

Akihiro Fujii

Akihiro Fujii